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The major goals of this project are to purify hantavirus proteins (Jonsson) for X-ray structure determination (Arnold). By defining the three-dimensional structures of the N and RDRP proteins of hantaviruses, our studies will afford a means to model drugs that specifically interfere with important stages of viral replication. Particularly, disrupting one or more functions of the RDRP is expected to result in effective disease treatment with little toxicity to host cells. In addition, our research will yield a high through-put in vitro assay for identification of new antiviral drugs. Together, these studies should lead toward effective therapeutic measures for controlling and treating hantaviral infections.

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Introduction

Statement of Work

This funded DOD work consists of a collaboration among Dr. Connie Schmaljohn at USAMRIID, Dr. Edward Arnold at the Center for Advanced Biotechnology and Medicine (CABM) at Rutgers University, and Dr. Colleen Jonsson at New Mexico State University (NMSU). The three laboratories cover a wide breadth of expertise in virology (Schmaljohn), biochemistry (Jonsson) and structural biology (Arnold). This report covers the funding provided to NMSU for the work of Dr. Jonsson and Arnold.

Milestones

1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU) - Year 1.
2. Define conditions for crystal production of SNV N protein (CABM) - Year 1.
3. Perform X-ray diffraction analysis of crystals of the SNV N protein to solve the three-dimensional structure (CABM) - Year 2.
4. Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP) (NMSU) and other soluble portions of the polymerase protein (USAMRIID) - Years 1 and 2.
5. Define conditions for crystal production of HTNV RDRP core domain (CABM) - Year 1.
6. Perform X-ray diffraction analysis of crystals of the HTNV RDRP core domain to solve the three-dimensional structure (CABM) - Year 2.
7. Produce and purify to at least 95% homogeneity the complete HTNV RDRP (NMSU) - Year 2.
8. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses (USAMRIID) - Year 1.
9. Complete studies to develop an assay for endonucleolytic cleavage of host mRNA using purified, expressed hantavirus polymerase protein (USAMRIID) - Year 2.

Significance and Rationale

Hantaviruses cause two serious human diseases, hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). Approximately 150,000 to 200,000 hospitalized cases of HFRS are reported each year throughout the world, with more than half typically occurring in China (Lee, 1996). The rest are found throughout other parts of Asia, Europe, Russia and Scandinavia. Mortality rates for HFRS vary from 1%-15%, depending in part on which hantavirus caused the infection. HPS is an emerging infectious disease that was first discovered in 1993 in the southwestern United States. Since then, more than 400 cases of HPS have been documented in North and South America, with mortality rates of approximately 40% (Childs and Bryan, 1998). Currently, licensed vaccines are not available for the prevention of HFRS or HPS. Therapeutic efforts are generally limited to supportive care, although studies performed in China on HFRS patients suggest that the drug ribavirin provides an improved prognosis when given early in the course of disease. Similar benefits have yet to be documented for HPS patients. No other antiviral drugs for treatment of hantaviral diseases have been identified. The studies described in this proposal are intended to provide a means for the rational design of antivirals for hantaviruses. In addition, because hantaviruses replicate in a manner similar to many other negative strand RNA viruses, our findings may be applicable for the design of effective therapeutics for other viral infections.

As a first step toward our goal of developing antiviral drugs for hantaviruses, we proposed to solve the three dimensional structures of the two key proteins needed for hantavirus replication: the RNA dependent RNA polymerase (RDRP) and the nucleocapsid protein (N). Because RDRPs are unique to viruses (i.e., there is no known cellular homologue), this class of enzyme is an attractive target for antiviral agents. The results of the proposed studies will allow us to model drugs that can interact with and disable specific portions of the RDRP. Moreover, the proposed work will generate assays that can be used for rapid screening of large numbers of antiviral drugs. Together, these efforts should lead toward identification of safe and effective means to treat not only known hantaviruses, but also those that are yet to emerge.

Body

I. Completed Studies

A. Milestone 1. Produce and purify to at least 95% homogeneity the Sin Nombre virus (SNV) nucleocapsid (N) protein (NMSU)

A.1. Purification of Recombinant Hantavirus N proteins

The following briefly describes the methods used to purify recombinant N protein (from soluble extracts) that will be used for the proposed studies. This section was more detailed in the first submission. Further details can be found in the manuscript: Jonsson, C.B., Gallegos, J., Fero, P., Xu, X. and C. S. Schmaljohn. Expression, Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein in *Escherichia coli*. *Protein Express. Purif.* 23:134-141

A.2 Expression and Extraction of the N Proteins of Hantaan and Sin Nombre Viruses

Expression vectors have been constructed that can generate large quantities of the N proteins from Sin Nombre (CC107) and Hantaan viruses (HTNV). The S-segment open reading frames (ORF) were cloned into the *Nde*I and *Xho*I sites of pET21b expression vectors (Novagen, Madison, WI), which generate a hexahistidine fusion at the C-terminus. We determined that the hexahistidine tag does not interfere with our RNA binding studies (Severson *et al.*, 1999). Initial studies showed that only a small fraction of the expressed N protein was soluble. Therefore, we optimized the expression strategy and extraction methods of the N protein to provide soluble protein with native extraction buffers. In general, *E. coli* BL21DE3 cells harboring the pET21b-N were grown overnight at 30°C, and then diluted 1:20 in Luria-Bertani (LB) medium containing 200 µg/ml of ampicillin. Following incubation of the diluted culture for 1 h at 30°C, IPTG to a final concentration of 1 mM was added to induce expression of the protein. After 1.5 h, the cells were harvested and resuspended in a solubilization buffer, SB (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 10 mM imidazole), and 0.25 mg/ml lysozyme. Cells were lysed and the soluble material was separated from the insoluble by centrifugation.

A.3 Purification of the N protein with Nickel NTA chromatography and SP Sepharose Chromatography.

Nickel Affinity Chromatography. The soluble portion of the protein extracts was applied to a 1 ml nickel NTA column preequilibrated in SB. The column was washed with SB containing 20 mM imidazole and the N protein was eluted with 250 mM imidazole. We have determined these conditions yield the greatest amount of N protein (7.5 mg/3.2 L). The protein material was dialyzed overnight at 4°C against two changes of 50 mM MES pH 6.2, 200 mM NaCl prior to loading onto a 1 ml Pharmacia SP sepharose column.

SP Sepharose Chromatography. Material isolated from soluble fractions were bound to a 1 ml Pharmacia SP sepharose column and a gradient from 200 mM to 1 M NaCl was run by the FPLC (Fig. 1A). The example shown in Fig. 1 is for the SNV CC107 N protein. The two peaks were examined by SDS PAGE (Fig. 1B). The first peak had a very low amount of N protein (Fig. 1B,

lanes 1 and 2), and therefore was assayed for its OD260 and OD280 values. The ratio was 1.7, which strongly suggests the presence of nucleic acids in these fractions. The major peak of N protein from both refolded and soluble preparation eluted at the same time, 9 min, at 660 mM NaCl concentration (Fig. 1A, peak 2, and Fig. 1B, lanes 3 and 4). This suggests that the refolded material has properties similar to the native material. At present, we estimate the isolated N protein has 95% or greater homogeneity.

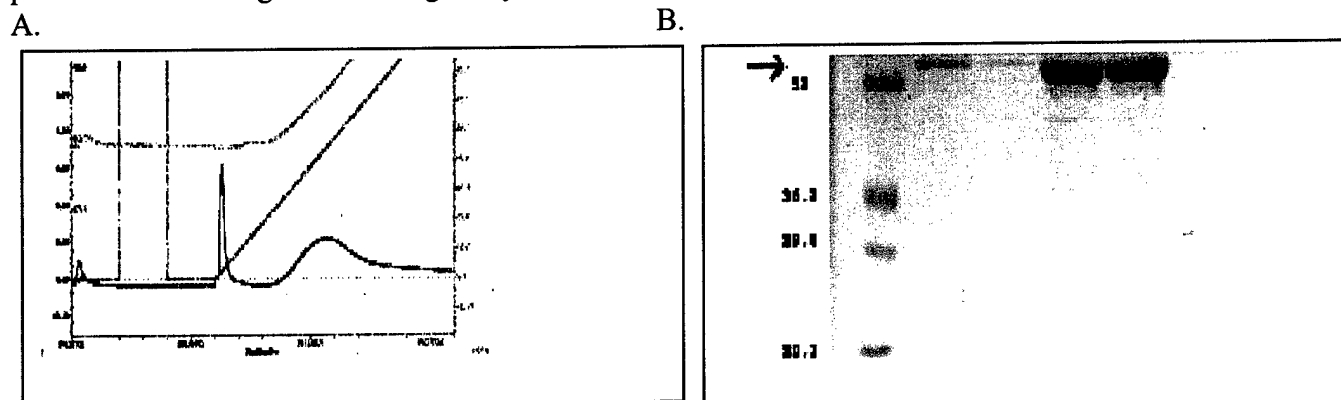


Fig. 1. SP sepharose chromatogram of CC107 N protein. (A). 100 ml of refolded N protein was loaded onto a 1 ml Pharmacia SP sepharose column. The column profile shown was run from 200 mM to 1 M NaCl. (B). Fractions from the two major peaks shown in the chromatogram in (A) are presented: Peak 1 -fractions 1 and 2 (Lanes 1 and 2); Peak 2- fractions 5 and 6 (Lanes 3 and 4).

A. 4 Mapping of the RNA Binding Domain of the Hantavirus N Protein (NMSU)

In order to capitalize on other subdomains that are more soluble, we must first identify the functional regions within the protein. Toward this goal, we have continued previous studies to define the RNA binding domain. To map the location of the RNA binding domain (RBD) of HTNV N protein, fourteen truncated constructs were prepared in the N-terminal, C-terminal or both regions of the N protein (Xu *et al.*, 2002). This work defined a minimal RBD between amino acid residues 175 to 217 that may extend into amino acids 217 to 249.

To further confirm the location of the RNA binding domain as well as provide insight into the use of peptides to explore the RBD, we have recently designed and purchased synthetic peptides of the RBD from Sigma-Genosys (Fig. 2). Dr. Severson has measured the RNA binding levels of each of the peptides with our filter binding methods. We are currently writing the manuscript for this and other data that detail the binding interaction.

N Peptide					binding
185-217	QSSMKAEEIT	PGRYRTAVCG	LYPAQIKARQ	MIS	wild type
195-217		PGRYRTAVCG	LYPAQIKARQ	MIS	excellent
206-217			LYPAQIKARQ	MIS	good
175-206	KHLYVSLPNA	QSSMKAEEIT	PGRYRTAVCG	LYP	good
175-196	KHLYVSLPNA	QSSMKAEEIT	PGR		weak
175-186	KHLYVSLPNA	QSS			poor

Fig. 2. Peptides synthesized within the putative HTNV RNA binding domain. A qualitative description of the binding activity is shown adjacent to each substrate.

Previously, we have measured and published the K_d for the full-length HTNVN protein with S-segment viral RNA substrates (53 ± 8 nM) (Severson, 1999). The largest peptide that Sigma-Genosys was able to synthesize was 185-217. Filter binding analysis of this peptide with the 5' end vRNA (1-39) substrate showed it to have a K_d of ~ 70 nM. The culmination of our previous work and these preliminary studies is embodied in this protein-RNA interaction—in that—the macromolecular interaction between these two biomolecules mimics the interaction characterized with the full-length protein and the full-length vRNA substrate.

- B. Milestones 2 and 3.** Define conditions for crystal production of SNV N protein (CABM) – Year 1. Perform X-ray diffraction analysis of crystals of the SNV N protein to solve the three-dimensional structure (CABM) - Year 2.

Samples of nucleocapsid protein appear monodisperse by gel filtration, anion exchange chromatography, and gel electrophoresis. The protein can be concentrated to between 10 and 20 mg/ml, which is an appropriate range for crystallization protocols. Limited crystallization experiments have been performed allowing the possible range of crystallization conditions to be narrowed down. Crystallization procedures utilize the hanging drop method, by which protein solutions are mixed with appropriate salts and precipitant solutions to induce crystal formation and growth. A trial of a large range of possible conditions has been tested but not crystals have yet formed.

The nucleocapsid proved to be quite difficult to approach by this technique. *Hence* over the past year we have enlisted the help of Dr. Markus Germann at Georgia State University. He has taken the HTNV N protein domain 175-300 and begun to examine its structure by circular dichroism and NMR. In addition he has made substantial progress on the structure of the end of the viral RNA, which is both a template for the polymerase and the encapsidation signal for the N protein,

- C. Milestones 4 and 7.** Produce and purify to at least 95% homogeneity the Hantaan virus (HTNV) RNA dependent RNA polymerase core domain (RDRP), full length RDRP (NMSU) and other soluble portions of the polymerase protein (USAMRIID)

C.1 Small Scale Expression

We have transformed the pET21b/HTNL into BLR DE3, HMS174 DE3, AD494 DE3, TUNER DE3, Rosetta DE3, and Origami DE3 and performed pilot scale expression studies. Briefly, we have inoculated a 5 ml overnight in LB/Amp200, grown at 37°C and 200 rpm for pilot expression study. Within 12-14 hours, 5 ml of the culture was added to 50 ml 2XYT/Amp100, grow at 30°C at 150 rpm for 1 hour. After 1 hour, 2-1 ml aliquots were taken; centrifuged and resuspended in 70 ul cracking buffer; labeled as uninduced whole cell protein extract. We use the other aliquot to test for protein solubility. The cultures were induced to 1 mM IPTG final concentration. After 1 hour and 3 hours, we took 2-1 ml aliquots and labeled as induced. The other aliquot was extracted into soluble and insoluble fractions. Protein samples were run on each sample on an SDS-PAGE gel (8%bottom-5%stacking); and a duplicate gel was transferred to PVDF membrane using a trans-blot semi dry Bio-Rad cell.

In conclusion the TUNER DE3 cells performed the best in the pilot-scale expression. The RdRp ran consistently between the 150 and 250 MW markers.

C.2 Optimizing Large Scale Expression

In our attempts to scale up the size of culture, we found that the expression of the RdRp was decreased. We have discovered that we can grow smaller cultures and hence we combine the cell pellets from 6 smaller cultures of 150 ml. This allowed the cells to grow to a higher cell density, but expression was still not very high and growth of the culture was also inhibited during induction. By comparing the cell pellet of the negative control (no insert—only vector transformed into Tuner cells) to the recombinant cells, the growth inhibition was determined to be caused by leaky expression of the RdRp. Leaky expression was inhibited by the addition of 0.5% glucose to the growth media. The glucose was removed by centrifugation before induction with IPTG. Glucose repression allowed the cells to grow to higher culture densities, so expression of the RdRp was elevated.

The RdRp was being produced, but was being degraded by proteases. Degradation was detected by western blot analysis of the negative control versus the recombinant cells. Degradation was decreased by growing the cells at 30 °C and inducing at room temperature. Growth and induction at temperatures lower than room temperature had no effect on the stability of the protein. Growing the cells in 2XLB (richer media) also stabilized the protein. Adding protease inhibitor to the extraction buffer also increased stability, but degradation continues to remain a problem.

Extraction of stable protein

1. The following buffers have been explored for the extraction of the RdRp:

1. 50 mM Na phosphate buffer—pH 8.0
0.3 M NaCl
20 mM BME
10 mM CHAPS
protease inhibitor
2. 8 M urea
50 mM Na phosphate buffer—pH 8.0
0.5 M NaCl
20 mM BME
10 mM CHAPS
0.3 M NaCl
50 mM Na phosphate buffer—pH 7.5
10% Glycerol
1% IGEPAL
protease inhibitor
3. 100 mM K Glutamate
20 mM HEPES—pH 7.6
1 mM DTT
1 % IGEPAL
5-20% glycerol
protease inhibitor
4. 0.4 M K Glutamate
20 mM HEPES—pH 7.6
1 mM DTT
1 mM EDTA, no EDTA
1 % IGEPAL
5-20% glycerol
6 M urea, no urea
protease inhibitor

All of these buffers extracted the protein, but the RdRp precipitated in each one immediately after extraction or after one freeze thaw cycle. Also, any attempt to concentrate the protein in these buffers made the protein precipitate out of solution.

2. Extraction followed by dialyzing into these buffers was also explored:

1. 100 mM HEPES—pH 7.6
100 mM NaCl
1 mM DTT
1 mM EDTA
10% glycerol
2. 200 mM K Glutamate
20 mM HEPES—pH 7.6
1 mM EDTA
1 mM DTT
1% IGEPAL
20% Glycerol

3. 20 mM HEPES—pH 7.6
200 mM K Glutamate
1 mM DTT
1 % IGEPAL
4. 20 mM HEPES—pH 7.6
200 mM K Glutamate
1 mM DTT
1 % Tween 200
5. 20 mM HEPES—pH 7.6
200 mM K Glutamate
1 mM DTT
1% Triton X
6. 20 mM HEPES—pH 7.6
200 mM K Glutamate
1 mM DTT
10 mM CHAPS
7. 20 mM HEPES—pH 7.6
200 mM K Glutamate
1 mM DTT
10% Glycerol

None of these met with success.

Finally, we have made a buffer with 10 mM K glutamate instead of the 100 mM required, and the RdRp was extracted and remained stable. It appears that each of the previous buffers contained too much salt. The following buffer extracts the RdRp in the soluble extract and allows it to remain soluble after freezing and thawing:

10 mM K Glutamate
20 mM HEPES—pH 7.6
1 mM DTT
1 % IGEPAL
20% glycerol
protease inhibitor

Current Extraction Protocol

Six 150 ml cultures of 2XLB/Amp 200/0.5% glucose were inoculated with 15 ml of media containing an overnight culture of pET21b/HTNL transformed into Tuner (DE3) cells. These cultures were grown at 30 °C and 220 RPM until the OD600 reached 0.4. The media containing glucose was removed by centrifugation at 5,000 x g for 5 min. The cells were resuspended in an equal amount of 2XLB/Amp 200/1 mM IPTG, and the cultures were induced at 25 °C and 150 RPM for 2.5 h. After induction, the cells were harvested by centrifugation at 5,000 x g for 5 min at 4 °C. The cells were resuspended in 15 ml of Buffer A (150 mM potassium glutamate, 1 x protease inhibitor, 20 mM HEPES-pH 7.6) and centrifuged at 13,000 x g for 10 min. The pellet was resuspended in 15 ml of Buffer B (20 mM HEPES-pH 7.6, 10 mM potassium glutamate, 1 mM DTT, 1 x protease inhibitor, 1 % IGEPAL, 20% glycerol) containing 150 mg of lysozyme.

The cells were lysed by douncing for 20 min on ice and sonication twice for 10 s at 40% power. The cell lysate was centrifuged at 13,000 x g for 30 min. The RdRp was extracted in the soluble portion.

Exploration of Purification Methods

1. Ni affinity chromatography. All of the RdRp was in the flow-through of the column. We thought this was due to the 6 X histidine tag being buried inside the protein, so I tried adding 2 M, 4 M, and 6 M urea to loosen the structure and make the histidine tag more accessible to the resin. None of these attempts worked, and the RdRp was always collected in the flow through.
2. Dialysis using high MWCO tubing and stirred cell dialysis. Neither the tubing nor the filter paper allowed smaller proteins through. When dialyzed for purification, we used the same buffer for dialysis that I used for extraction. (Buffers listed above)
3. Ammonium sulfate precipitation. Using dry ammonium sulfate, 20%, 40% and 60% ammonium sulfate was added to the soluble extract. After adding the ammonium sulfate, the solution rested on ice with occasional stirring for 20 min. The solutions were centrifuged at 13,000 x g for 15 min to collect the precipitated proteins. The RdRp precipitated at 20% concentration and easily resuspended in 1 ml of Buffer B. While this did not purify the protein much, it definitely concentrated the RdRp. Trying concentrations of ammonium sulfate that are closer to 20% (10%, 15%, 20% and 25%) may allow more selective precipitation.
4. Size Exclusion Chromatography. Currently, we are exploring concentrating the protein using centriprep devices from Millipore that have a high MWCO and may be used on highly concentrated samples, such as the cell lysate. Since we have found a stable buffer for the RdRp, concentrating the sample should not be as difficult as before. After concentrating (and hopefully clearing smaller proteins from the lysate), we will run the extract over a Sepharose 6 gel filtration column. SEC seems promising, since the RdRp is larger than any of the bacterial proteins.

C.3 Produce and purify to at least 95% homogeneity the complete HTNV RdRp (NMSU). Development of Methods to Overexpress and Purify the HTNV RdRp from E. coli

The open reading frame of the HTNV L-segment was cloned into the bacterial expression vector pET21b, sequenced in both directions, and expressed as a C-terminal hexahistidine fusion protein. At the N-terminal end, a T7 tag was incorporated for visualization by immunoblot. Small scale expression of the RdRp, examined in six bacterial cell lines, was highest in Tuner(DE3) cells. SDS-PAGE revealed a band at 250 kDa, which was confirmed on the corresponding immunoblot using anti-T7 tag antibodies.

Several conditions have been explored for expression and extraction of the RdRp as discussed earlier. In the following, our most recent protocol is discussed. A 150 ml culture of

LB/Amp100/0.5% glucose was inoculated with a 15 ml overnight culture of Tuner (DE3) *E.coli* cells transformed with the recombinant pET21b/Hantaan L segment plasmid. This culture was allowed to grow at 37 °C and 225 RPM until the culture density reached an optical density of 0.55 at an absorbance of 600 nm. The media was removed by centrifugation at 5,000 x g for 5 min, and the cells were resuspended in 150 ml of LB/Amp 100 containing 1 mM isopropylthiogalactosidase (IPTG). The cells were incubated at 25 °C and 150 RPM for 2.5 h, and then harvested by centrifugation at 5,000 x g for 5 min. The cell pellet was resuspended in 10 ml of 20 mM HEPES (pH 7.6) and 150 mM potassium glutamate. The cells were again harvested by centrifugation, and then resuspended in 10 ml of 20 mM HEPES (pH 7.6), 100 mM potassium glutamate, 5 mM MgAc₂, 1 mM DTT, and 1% IPEGAL. The cells were lysed using lysozyme, mechanical lysis, and sonication. The lysate was centrifuged at 13,000 x g for 30 min, and then the pellet was resuspended in 10 ml of the same buffer. The centrifugation was repeated, and the final pellet was resuspended in 10 ml of 0.4 M potassium glutamate, 20 mM HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 1% IPEGAL, and 10% glycerol. The centrifugation was repeated, and the pellet was resuspended in 10 ml of 0.4 M potassium glutamate, 20 mM HEPES (pH 7.6), 1 mM EDTA, 1 mM DTT, 1% IPEGAL, 10% glycerol, and 4 M urea. The mixture was incubated on ice for 15 min and then centrifuged at 13,000 x g for 30 min. The protein extract was incubated with 0.5 ml of Ni-NTA resin for 2.5 h, and then loaded onto a 1 ml column. The column was washed with 5 ml of the extraction buffer containing 15 mM imidazole. The protein was eluted from the column in five 1.5 ml fractions using the extraction buffer containing 250 mM imidazole.

Key Research Accomplishments

- Method for purification of soluble, homogenous N protein from *E. coli*
- Defined the biologically relevant domains of N for future structural work
- Five additional clones for RDRP expression in *E. coli*
- Expression of three RDRP clones in *E. coli*
- Purification protocol for RDRP clones in *E. coli*
- Production of monoclonal antibodies to RDRP (full length) *in progress*
- Development of an assay for endonucleolytic cleavage of host mRNA using infectious hantaviruses
- Defined conditions for crystal production of SNV N protein
- Initiated collaboration with Dr. M. Germann at Georgia State to pursue NMR structure of hantavirus proteins

Reportable Outcomes

Manuscripts

Severson, W.E., Xu, X., Kuhn, M., Chapman, S., Senutovitch, N. and C. B. Jonsson: Amino acids essential to binding activity of the RNA binding domain of the Hantaan N protein. Manuscript to be submitted in June 2003 to J. Virology.

Leon, L., Thokala, M, and Jonsson, C. Expression of the 246 kDa RNA dependent RNA polymerase in Escherichia coli. Manuscript to be submitted in July/August 2003 to PNAS.

Meeting Abstracts, May 1, 2000 – April 30, 2003

American Society of Virology, KY, 20-24 July 2002, Characterization of the RNA Binding Domain of the Hantaan Virus N Protein, Colleen B. Jonsson, oral presentation

FASEB – American Society for Biochemistry and Molecular Biology, April 19-24, 2003, San Diego, Purification and Characterization of Hantaan Virus 246 kDa RNA Dependent RNA Polymerase (RdRp), Lisa Leon, poster presentation. Awarded first prize in Undergraduate poster competition.

Conclusions

Substantial progress has been made toward the original stated goals. While certain aspects of the project were difficult, we have modified our approach to solve these hurdles, such as the use of NMR rather than X-ray. The DOD support of these studies has permitted rapid growth in an area that has grown in national importance since the funding of this grant. Hantaviruses represent an important and growing source of disease emergence in both established and developing countries. Furthermore, the New World hantaviruses have been listed as potential biological weapons because of their lethality to humans and high infectivity by the aerosol route. Effective vaccines and antivirals for the treatment or prophylaxis of hantaviral infections are currently unavailable for hantavirus infection. Intensive vaccine efforts are in progress at USAMRIID as hantaviruses such as HTNV are considered an important infectious disease to the military. The studies supported by DOD in this grant have helped establish the necessary tools and approaches and biological understanding to develop additional therapeutics that will complement the on-going vaccine efforts. Importantly, our goal is to continue to design and develop derivatives of these drugs that will preferentially interact with the viral polymerase. In addition, because hantaviruses replicate in a manner similar to many other negative strand RNA viruses, our findings may be applicable for the design of effective therapeutics for other viral infections such as Crimean-Congo hemorrhagic fever and Rift Valley fever viruses.